

# *In Vitro* and *In Vivo* Biological Effects of Novel Arylimidamide Derivatives against *Trypanosoma cruzi*

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Chagas disease (CD), a neglected tropical disease caused by *Trypanosoma cruzi*, remains a serious public health problem in several Latin American countries. The available chemotherapies for CD have limited efficacy and exhibit undesirable side effects. Aromatic diamidines and arylimidamides (AIAs) have shown broad-spectrum activity against intracellular parasites, including *T. cruzi*. Therefore, our aim was to evaluate the biological activity of eight novel AIAs (16DAP002, 16SAB079, 18SAB075, 23SMB022, 23SMB026, 23SMB054, 26SMB070, and 27SMB009) against experimental models of *T. cruzi* infection *in vitro* and *in vivo*. Our data show that none of the compounds induced a loss of cellular viability up to 32  $\mu$ M. Two AIAs, 18SAB075 and 16DAP002, exhibited good *in vitro* activity against different parasite strains (Y and Tulahuen) and against the two relevant forms of the parasite for mammalian hosts. Due to the excellent selective indexes of 18SAB075, this AIA was moved to *in vivo* tests for acute toxicity and parasite efficacy; nontoxic doses (no-observed-adverse-effect level [NOAEL], 50 mg/kg) were employed in the tests for parasite efficacy. In experimental models of acute *T. cruzi* infection, 18SAB075 reduced parasitemia levels only up to 50% and led to 40% protection against mortality (at 5 mg/kg of body weight), being less effective than the reference drug, benznidazole.

Chagas disease (CD) is a neglected tropical disease caused by the intracellular flagellate protozoan *Trypanosoma cruzi*, which presents a complex life cycle with distinct morphological stages in its obligatory passage through vertebrate and invertebrate hosts (1). Currently, there are approximately 10 million infected individuals in areas of Latin America where CD is endemic, and many reports also point to the occurrence of CD in geographical areas where it is not endemic, such as the United States and Europe, mainly attributed to migration of infected people (2–6). CD can be transmitted by *Triatominae* insect feces, blood transfusion, organ transplantation, and laboratory accidents and through oral and congenital routes (7, 8). This pathology has two successive phases, a short, acute phase characterized by a patent parasitemia, followed by a chronic phase in which most of the infected individuals remain asymptomatic (indeterminate form), but about one-third may later manifest cardiac and/or digestive complications, developed progressively for years or decades after infection (9, 10). Benznidazole (Bz) and nifurtimox, introduced into clinical therapy about 40 years ago, are the only available drugs. Both have several shortcomings related to their required long periods of treatment, high toxicity, variable results, and low efficacy during the chronic phase, justifying the identification of novel therapies (11–13). Aromatic diamidines and analogues exhibit broad-spectrum activity against pathogenic microorganisms, including *T. cruzi* (14). Among the different tested derivatives of amidine compounds, the most effective against *T. cruzi* have been the bis-arylimidamides (AIAs) like DB766 (15) and DB1831 (16). Thus, in this study, we assayed the biological activities of eight novel AIAs (16DAP002, 16SAB079, 18SAB075, 23SMB022, 23SMB026, 23SMB054, 26SMB070, and 27SMB009) against experimental models of *T. cruzi* infection *in vitro* and *in vivo*, using different parasite strains, and also explored their toxicities in cardiac cell cultures and mouse models of acute toxicity.

## MATERIALS AND METHODS

**Synthesis of the arylimidamides.** The eight arylimidamides, all isolated as their hydrochloride salts, were prepared by three general methods (see the supplemental material). Briefly, phenylimidamides 16DAP022 and 16SAB079 (Fig. 1) were prepared by the reaction of 2,7-diaminocarbazole (prepared by reduction of 2,7-dinitrocarbazole) (17) or *m*-xylylenediamine with (2-naphthyl)methyl benzothioimidate hydrobromide (18). The 2-pyridylimidamide 18SAB075 (Fig. 2) was prepared by the reaction of 4,4'-diaminodiphenylacetylene (19, 20) with 2-cyanopyridine by the method of Lange et al. (21) but using lithium (rather than sodium) bis(trimethylsilyl)amide. The reaction of the methyl imidate derivatives of 2- or 4-cyanopyridine with the appropriate  $\alpha,\omega$ -diamines or spermidine gave 2-pyridylimidamides 23SMB022, 23SMB026, and 23SMB054 and 4-pyridylimidamides 26SMB070 and 27SMB009 (Fig. 3).

**Mammalian cell cultures.** For the *in vitro* analysis of compound toxicity and effects against intracellular parasites (Y strain), primary cultures of embryonic cardiomyocytes (CM) were obtained from Swiss mice as previously reported (22). After purification, the CM were seeded at a density of  $0.2 \times 10^6$  and  $0.05 \times 10^6$  cells/well, respectively, in 24- and 96-well microplates containing gelatin-coated coverslips. The cultures were then maintained at 37°C in Dulbecco's modified medium supplemented with 10% horse serum, 5% fetal bovine serum, 2.5 mM CaCl<sub>2</sub>, 1 mM L-glutamine, and 2% chicken embryo extract (DMEM). For the further analysis of the effect on intracellular parasites of the Tulahuen strain

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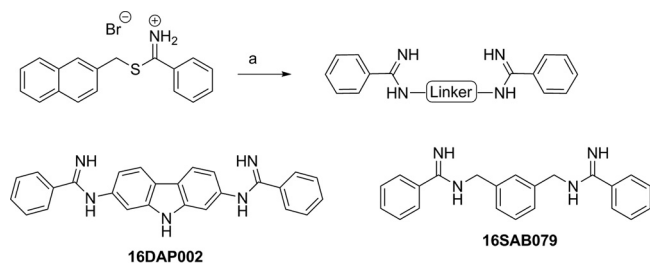
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**FIG 1** Synthesis of 16DAP002 and 16SAB079. Reagents and conditions: (a) appropriate diamine, ethanol (plus acetonitrile for 16DAP002), 0 to 25°C, overnight (66 to 71%).

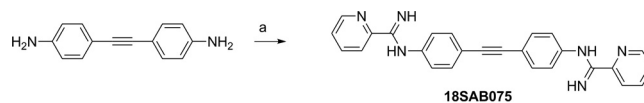
(parasites expressing the *Escherichia coli*  $\beta$ -galactosidase gene), monolayers of mouse L929 fibroblasts were cultivated ( $4 \times 10^3$  cells/well in 96-well microplates) at 37°C in RPMI 1640 medium (pH 7.2 to 7.4) without phenol red (Gibco BRL) supplemented with 10% fetal bovine serum (FBS) and 2 mM glutamine (RPMIS), as reported previously (23).

**Parasites.** Bloodstream trypomastigote (BT) forms of the Y strain were obtained from the blood samples of infected albino Swiss mice at the peak of parasitemia. The purified parasites were resuspended in Eagle's medium modified by Dulbecco's medium (DME) supplemented with 10% FBS (DMES) as reported previously (22). The effect on the intracellular forms was investigated by both the infection of L929 cell lineages with tissue culture-derived trypomastigotes (Tulahuen strain expressing the *E. coli*  $\beta$ -galactosidase gene) and the infection of CM with bloodstream trypomastigotes (Y strain) using a 10:1 ratio, following previously established protocols (15, 23). Stock solutions were prepared in dimethyl sulfoxide (DMSO) with the final concentrations of the solvent never exceeding 0.6% and 10% for *in vitro* and *in vivo* analysis, respectively, which did not exert any toxicity (data not shown). Benznidazole (Bz) (2-nitroimidazole; Laboratório Farmacêutico do Estado de Pernambuco [LAFPE], Brazil) was used as a reference drug.

**Cytotoxicity *in vitro* tests.** CM were incubated for 24 h at 37°C with different concentrations of each compound (up to 96  $\mu$ M) diluted in DMEM (without phenol red), their morphology and spontaneous contractibility were evaluated by light microscopy, and then their cellular viability was determined by the alamarBlue assay. For this colorimetric bioassay, 10  $\mu$ l alamarBlue (Invitrogen) was added to each well, and the plate was further incubated for 24 h, after which the absorbances at 570 and 600 nm were measured. As negative controls, alamarBlue assays were also performed in the absence of cells, using only DMEM and DMEM containing each tested compound (at 96  $\mu$ M). The results are expressed as the percent differences in the decreases between compound-treated and vehicle-treated cells by following the manufacturer's instructions and the EC<sub>50</sub> value corresponds to the concentration that reduces the cellular viability by 50% (23).

**Trypanocidal analysis.** Bloodstream trypomastigotes of the Y strain ( $5 \times 10^6$ /ml) were incubated for 24 h at 37°C in RPMI medium in the presence or absence of serial dilutions of the compounds (0 to 32  $\mu$ M). After compound incubation, the parasite death rates were determined by light microscopy through direct quantification of the numbers of live parasites using a Neubauer chamber, and the EC<sub>50</sub> (compound concentration that reduces 50% of the number of parasites) was then calculated.

For the assays on intracellular forms, different protocols were performed with the Y and Tulahuen strains. For Tulahuen-infected L929 cells, the cultures were exposed to 1.0  $\mu$ g/ml diluted in RPMIS, and those compounds that presented  $\geq 50\%$  reductions in the parasite infection index were further screened using increasing concentrations with the aim of determining the EC<sub>50</sub>s (23). After 96 h of compound incubation at 37°C, chlorophenol red glycoside (500  $\mu$ M) in 0.5% Nonidet P40 was added to each well, and the plate was incubated for 18 h at 37°C. Next, the absorbance was measured at 570 nm. Uninfected and infected cultures submitted to vehicle and Bz exposure were tested in parallel. The results



**FIG 2** Synthesis of 18SAB075. Reagents and conditions: (a) 2-cyanopyridine, lithium bis(trimethylsilyl)amide, tetrahydrofuran, 25°C, 2 days, (16%).

are expressed as the percentage of *T. cruzi* growth inhibition in compound-tested cells compared to that in the infected cells and untreated cells (23).

For the analysis of the effect against intracellular amastigotes from the Y strain, after 24 h of parasite-host cell interaction, the infected cultures were washed to remove free parasites and then incubated for another 48 h with increasing concentrations of the test compounds. CM were maintained at 37°C in an atmosphere of 5% CO<sub>2</sub> and air, and the medium was replaced every 24 h. Then, untreated and treated infected CM were fixed and stained with Giemsa solution, and the mean numbers of infected host cells and of parasites per infected cell were scored as reported previously (16). Only characteristic *T. cruzi* nuclei and kinetoplasts were counted as surviving parasites since irregular structures might mean parasites undergoing death. The compound activity was estimated by calculating the infection index (II) (the percentage of infected cells times the average number of intracellular amastigotes per infected host cell) (24). Triplicate assays were run on the same plate, and at least two assays were performed in each analysis.

**Mouse acute toxicity.** In order to determine the no-observed-adverse-effect level (NOAEL), each dose of the tested compounds (12.5 to 200 mg/kg of body weight) was injected by the intraperitoneal (i.p.) route individually in Swiss Webster female mice (20 to 23 g). On days 2 and 3, mice were inspected for toxic and subtoxic symptoms according to the Organization for Economic Co-operation and Development (OECD) guidelines. Forty-eight hours after compound injection, the NOAEL values were determined, and plasma biochemical analysis was performed for alanine aminotransferase (ALT), urea, and creatine kinase (CK) as reported previously (16).

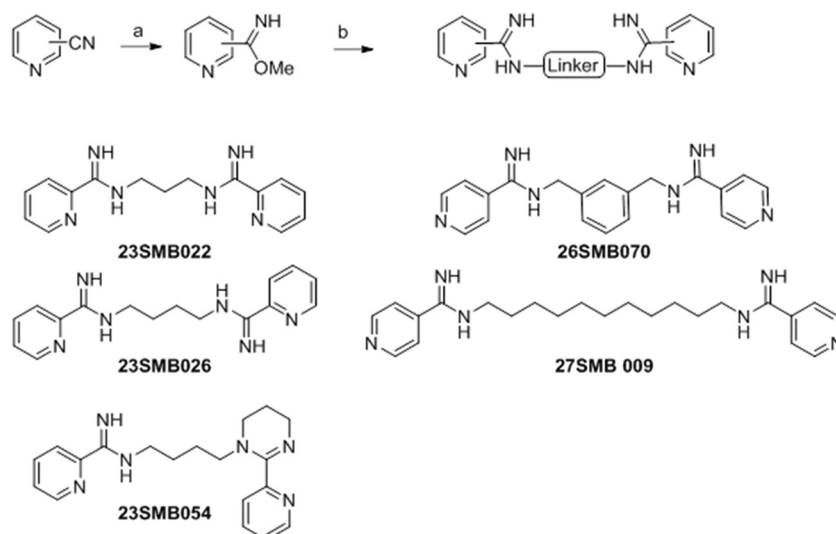
**Mouse infection and treatment schemes.** Male Swiss mice were obtained from the Fundação Oswaldo Cruz (FIOCRUZ) animal facilities (Rio de Janeiro, Brazil). Mice were housed at a maximum of 6 per cage and kept in a conventional room at 20 to 24°C under a 12 h/12 h light/dark cycle. The animals were provided with sterilized water and chow *ad libitum*. Infection was performed by i.p. injection of  $10^4$  bloodstream trypomastigotes (Y strain). The animals (18 to 21 g) were divided into the following groups: uninfected (noninfected and untreated), untreated (infected with *T. cruzi* but treated only with vehicle), and treated (infected and treated i.p. with 0.5 to 20 mg/kg/day test compound [up to 0.2 ml] or with 100 mg/kg/day Bz orally [p.o.]). The mouse treatment started at 5 days postinfection (dpi) followed by (i) a 1-s dose at 8 dpi or (ii) five consecutive daily doses. For Bz treatment, infected mice received a 0.2-ml oral dose (gavage) following the same therapeutic schemes as described above (15).

**Parasitemia, mortality rates, and ponderal curve analysis.** Parasitemia was individually checked by direct microscopic counting of parasites in 5  $\mu$ l of blood, as described before (25). Body weight was evaluated weekly, and mortality was checked daily until 30 days posttreatment and expressed as a percentage of cumulative mortality (%CM) (15).

**Ethics.** All procedures were carried out in accordance with the guidelines established by the FIOCRUZ Committee of Ethics for the Use of Animals (CEUA LW-16/2013).

## RESULTS

The screening of the eight novel arylimidamides (Fig. 1 to 3) against bloodstream trypomastigotes (Y strain) revealed that after 2 h of incubation at 37°C, 18SAB075 and 16DAP002 showed considerable activity, displaying EC<sub>50</sub>s of 11 and 14  $\mu$ M (Table 1).



**FIG 3** Synthesis of 23SMB022, 23SMB026, 23SMB054, 26SMB070, and 27SMB009. Reagents and conditions: (a) sodium methoxide, methanol, 25°C, 1 week; (b) appropriate  $\alpha,\omega$ -diamine (or spermidine for 23SMB054), 4 M HCl in dioxane, methanol, 25°C (5 to 92%).

After 24 h of incubation, both were more effective than Bz, displaying  $EC_{50}$ s of 0.3 and 3  $\mu$ M, respectively, with the former molecule exhibiting about 43-fold higher trypanocidal effect than the reference drug Bz (Table 1). *In vitro* analysis of cytotoxicity on cardiac cell cultures performed by the alamarBlue assay showed that up to 32  $\mu$ M, none of the studied compounds caused loss of cellular viability (Table 1). An alamarBlue assay performed in the absence of cells, with DMEM containing or not containing each tested compound (at 96  $\mu$ M), showed no alterations in the absorbance measurements (data not shown). When tested on *T. cruzi*-infected host cells (the Tulahuen strain transfected with a  $\beta$ -galactosidase gene) under a standardized screening protocol using a fixed concentration of 1  $\mu$ g/ml (23), again both 16DAP002 and 18SAB075 were effective (Table 2). Next, the two AIAs were screened against Tulahuen-infected host cells in order to determine the  $EC_{50}$ s. The findings showed that 18SAB075 was the most active with values of 1.5  $\mu$ M after 96 h of incubation, comparable to those for Bz and 16DAP002 (Table 3). Further analysis of the

**TABLE 1** Trypanocidal activity ( $EC_{50}$ ) of the studied arylimidamides against bloodstream trypomastigotes of *T. cruzi* (Y strain) and their selectivity index related to cardiomyocytes ( $EC_{50}$ )

Compound	$EC_{50}$ (mean $\pm$ SD) ( $\mu$ M) in:			SI (24 h) <sup>a</sup>
	Trypomastigotes		Cardiomyocytes (24 h)	
	2 h	24 h		
18SAB075	11 $\pm$ 1	0.3 $\pm$ 0	>32	>106
16DAP002	14 $\pm$ 3	3 $\pm$ 1	>32	>11
27SMB009	>100	85 $\pm$ 21	>32	>0.4
23SMB054	>100	>100	>32	
23SMB022	>100	>100	>32	
23SMB026	>100	>100	>32	
26SMB070	>100	>100	>32	
16SAB079	>100	>100	>32	
Benznidazole	>100	13 $\pm$ 2 <sup>b</sup>	1,000	77

<sup>a</sup> Treatment for 24 h at 37°C. Selectivity index (SI) =  $EC_{50}$  for cardiomyocytes/ $EC_{50}$  for parasites.

<sup>b</sup> Value from da Silva et al. (16).

effect of 18SAB075 against *T. cruzi*-infected cardiac cells using another strain (Y strain) confirmed the promising activity ( $EC_{50}$  = 0.9  $\mu$ M) of this AIA, which was especially notable in the data for the mean number of infected cardiac cells when the infected cultures were incubated with concentrations up to 32  $\mu$ M without causing loss of host cell viability (Fig. 4A and B). Although no loss of viability was noted up to  $\leq$ 32  $\mu$ M as seen by the alamarBlue method, light microscopy analysis demonstrated that 16SAB002 at concentrations  $>$ 3.5  $\mu$ M induced cellular vacuolization and impaired the contractibility of the cardiac cells *in vitro*. Also, no effect on the levels of *T. cruzi* infection was observed when nontoxic doses ( $\leq$ 3.5  $\mu$ M) of this AIA were tested (data not shown).

Next, due to the higher selectivity indexes of 18SAB075 on bloodstream trypomastigotes ( $>$ 106; Table 1) and on intracellular forms (40; Fig. 4), this compound was moved to *in vivo* analysis.

Our first *in vivo* step was to evaluate aspects of the acute toxicity of 18SAB075, aiming to determine its NOAEL value. The administration of this AIA via the i.p. route to Swiss female mice (12.5 to 200 mg/kg) followed for 48 h showed that none of the doses caused

**TABLE 2** Activity of arylimidamides (at 1  $\mu$ g/ml) against intracellular forms of *T. cruzi* (Tulahuen strain transfected with  $\beta$ -galactosidase) after treatment for 96 h at 37°C

Compound	Inhibition of the host cell infection (mean $\pm$ SD) (%)
16DAP002	58 $\pm$ 31
18SAB075	50 $\pm$ 7
26SMB070	15 $\pm$ 8
16SAB079	4 $\pm$ 5
23SMB054	3 $\pm$ 4
23SMB022	1 $\pm$ 1
27SMB009	0 $\pm$ 0
23SMB026	0 $\pm$ 0
Benznidazole	82 $\pm$ 3

**TABLE 3** *In vitro* effect ( $EC_{50}$ s) of arylimidamides against intracellular forms of *T. cruzi* (Tulahuen strain transfected with  $\beta$ -galactosidase) after treatment for 96 h at 37°C

Compound	$EC_{50}$ (mean $\pm$ SD) ( $\mu$ M)
16DAP002	4.1 $\pm$ 1.8
18SAB075	1.5 $\pm$ 0.2
Benznidazole	2.6 $\pm$ 0.8

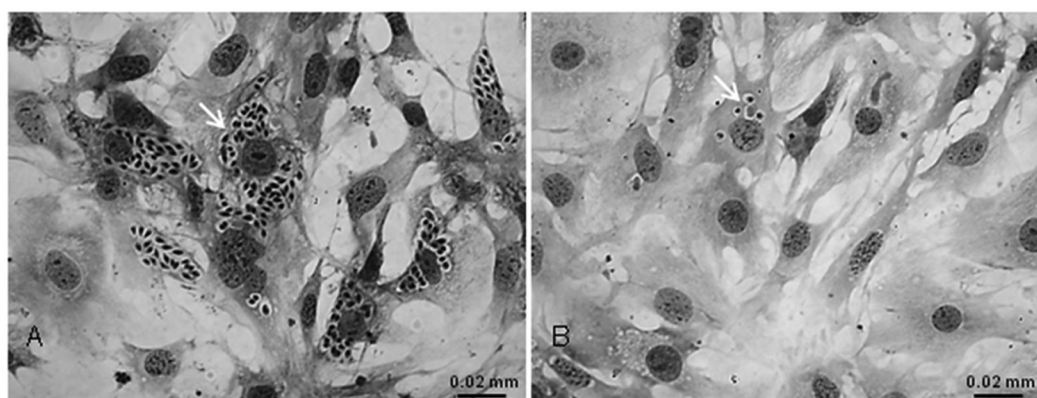
death. Up to 50 mg/kg, no alteration in animal behavior (compared to that of vehicle-treated mice) was found. However, increased doses induced visible neurological disorders like tremors, ataxia, and hyperactivity (data not shown). The gross pathological examination performed at 48 h after drug administration showed that 200 mg/kg induced hemorrhagic hepatic signs (data not shown). This hepatic injury was confirmed by the biochemical plasma analysis of the higher dose as increased levels of aspartate transaminase (AST) and ALT were found compared to those for the animals from the other groups (data not shown). Then, the efficacy of 18SAB075 was tested in Swiss male mice inoculated with  $10^4$  bloodstream parasites using a therapeutic scheme employing doses administered once a day, at 5 and 8 dpi (Fig. 5) that correspond to the onset and parasitemia peaks, respectively, in this animal model (15). Only parasitemia-positive mice were used, and Bz treatment was run in parallel using standard protocol (100 mg/kg p.o.), following the same therapeutic scheme as described above (Fig. 5). Although 20 and 10 mg/kg did not reduce or only slightly impaired (20%), respectively, the parasitemia levels, the lower dose (5 mg/kg) resulted in a 50% decrease in the parasitemia peak decrease, also leading to 40% animal survival while all the other mice (except for the Bz group) died (Fig. 5).

Then, aiming to allow a longer plasma exposure of the compound but taking into consideration the use of maximum non-toxic doses (up to 50 mg/kg), we performed additional studies, providing the AIA for 5 daily consecutive days, starting at 5 dpi (Fig. 6). As expected for this experimental mouse model of *T. cruzi* acute infection, infected and vehicle-treated animals presented high parasitemia and no animal survival (Fig. 5 and 6). When 18SAB075 was administered via the i.p. route, none of the doses reduced the parasitemia or protected against mortality. As expected, Bz completely suppressed the parasitemia and conferred 100% survival (Fig. 6). Also, none of the doses were able to protect the animals against the body weight loss (data not shown) induced by the *T. cruzi* infection in this experimental acute model (26).

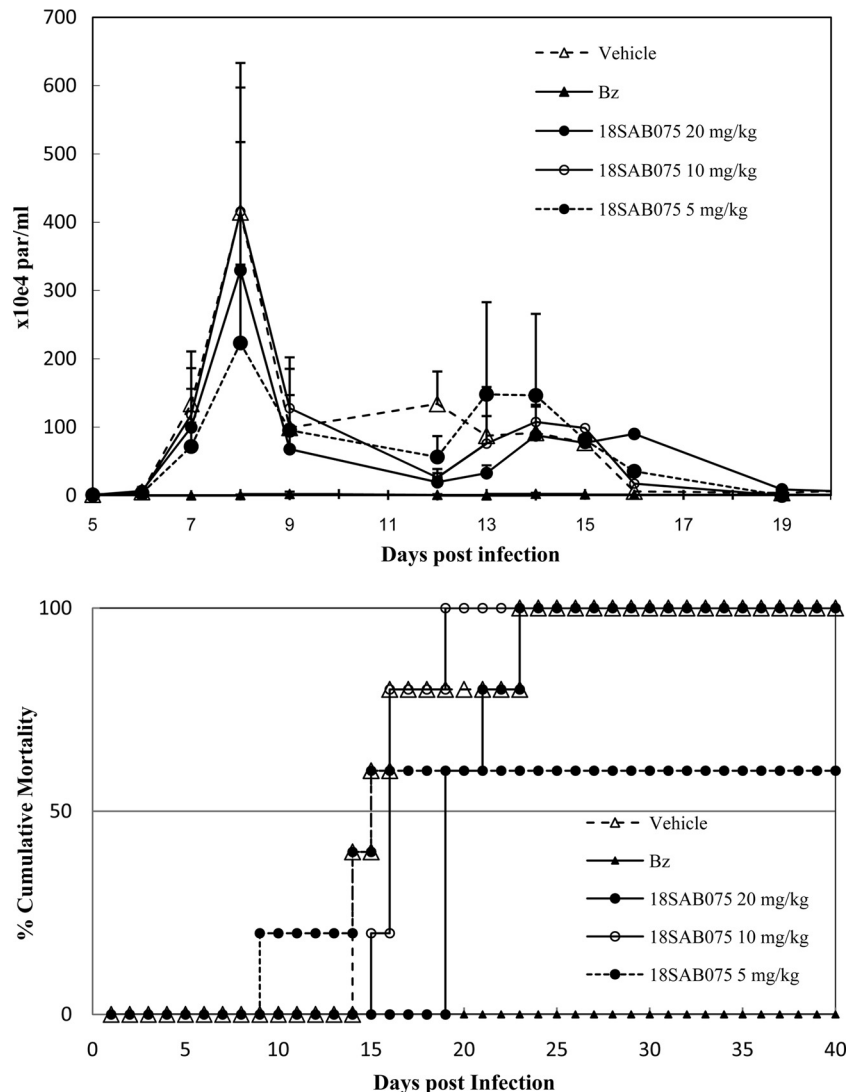
## DISCUSSION

Structural variations in the presently available AIAs include seven different linkers between the two AIA moieties and three different outer aromatic rings. Those molecules containing multiple or fused aromatic rings in their linkers were active against the bloodstream and intracellular parasites. The most active compound, 18SAB075, contains a diphenylacetylene linker, which offers extended conjugation. The second most active compound, 16DAP002, contains a carbazole system as its linker, which also offers extended conjugation but a more rigid conformation due to the fused rings. Both compounds displayed AIA nitrogen atoms attached directly to aromatic rings. The xylene derivative 26SMB070, which was less active, bears an aromatic ring in its linker, but the nitrogen atoms are attached to aliphatic carbons. Analogues of the two most active compounds containing the same linkers but different outer rings were not included in this study.

18SAB075	$EC_{50}$ ( $\mu$ M)	SI
% Host cell Infection	0.8 $\pm$ 0.1	40
Mean number of parasite/per infected host cell	6.0 $\pm$ 2.0	5
Infection Index	0.9 $\pm$ 0.1	36



**FIG 4** Activity ( $EC_{50}$ s) and selective index (SI) of 18SAB075 against intracellular forms of *T. cruzi* (Y strain) after treatment for 48 h at 37°C. (A) *T. cruzi*-infected cardiac cells incubated with vehicle. (B) *T. cruzi*-infected cardiac cells incubated with 32  $\mu$ M 18SAB075. The arrows point to intracellular parasites. Infection index, host cell infection  $\times$  number of parasites/infected host cell, as reported in Batista et al. (15).



**FIG 5** *In vivo* effect of 18SAB075 on *T. cruzi* infection using male Swiss mice inoculated with  $10^4$  bloodstream trypomastigotes (Y strain). The activities of 5, 10, and 20 mg/kg/day 18SAB075 (i.p.) and of benznidazole (100 mg/kg/day by gavage) given at 5 and 8 dpi was evaluated through parasitemia levels (top) and cumulative mortality (bottom).

Chemotherapeutic options for treating chagasic patients currently depend on only two nitro derivative drugs, namely, benznidazole (2-nitroimidazole; Laboratório Farmacêutico do Estado de Pernambuco [LAFEPE], Brazil) and nifurtimox (5-nitrofurán, Bayer 2502; Bayer, Germany) (27). Both cause severe side effects, resulting in discontinuation of the treatment and low effectiveness, especially in the later chronic phase of the disease, demanding the identification of novel drugs to treat this devastating chronic pathology. However, there is a lack of interest from most pharmaceutical companies for discovery of new anti-*T. cruzi* agents mainly due to the long time and high costs associated with the drug pipeline for this and other neglected parasitic illnesses that afflict millions of the poorest people worldwide (28). In this context, our aim was to explore the possibility of finding new amidine derivatives with considerable activity against *T. cruzi* in *vitro* and *in vivo* which possess the potential to act as clinical candidates against this neglected parasitic disease. In this vein, eight novel AIAs were screened against the bloodstream and intracellu-

lar forms of the parasite and two of them, 18SAB075 and 16DAP002, demonstrated higher activity, with  $EC_{50}$ s of  $<4 \mu\text{M}$ . The two compounds were effective against different strains (Y and Tulahuen), and due to its high selectivity, 18SAB075 was further examined in *in vivo* analyses for preliminary ascertainment of acute toxicities and for efficacy determination using an acute mouse model of *T. cruzi* infection (male Swiss mice infected with Y strain). The follow-up of acute toxicity signs for 48 h after 18SAB075 administration via the i.p. route showed that although at up to 50 mg/kg, no alterations were noticed, considerable toxic side effects like ataxia, hyperactivity, and tremors were found with doses of 100 and 200 mg/kg. The gross pathological examination confirmed that the higher dose also induced hemorrhagic hepatic signs that were corroborated by increased plasma levels of hepatic markers like AST. Thus, efficacy analysis was further explored using only doses  $\leq 20$  mg/kg/day and never exceeding cumulative doses of 50 mg/kg. Bz was included as a reference drug, given at its effective dose of 100 mg/kg/day (23). The i.p. administration of

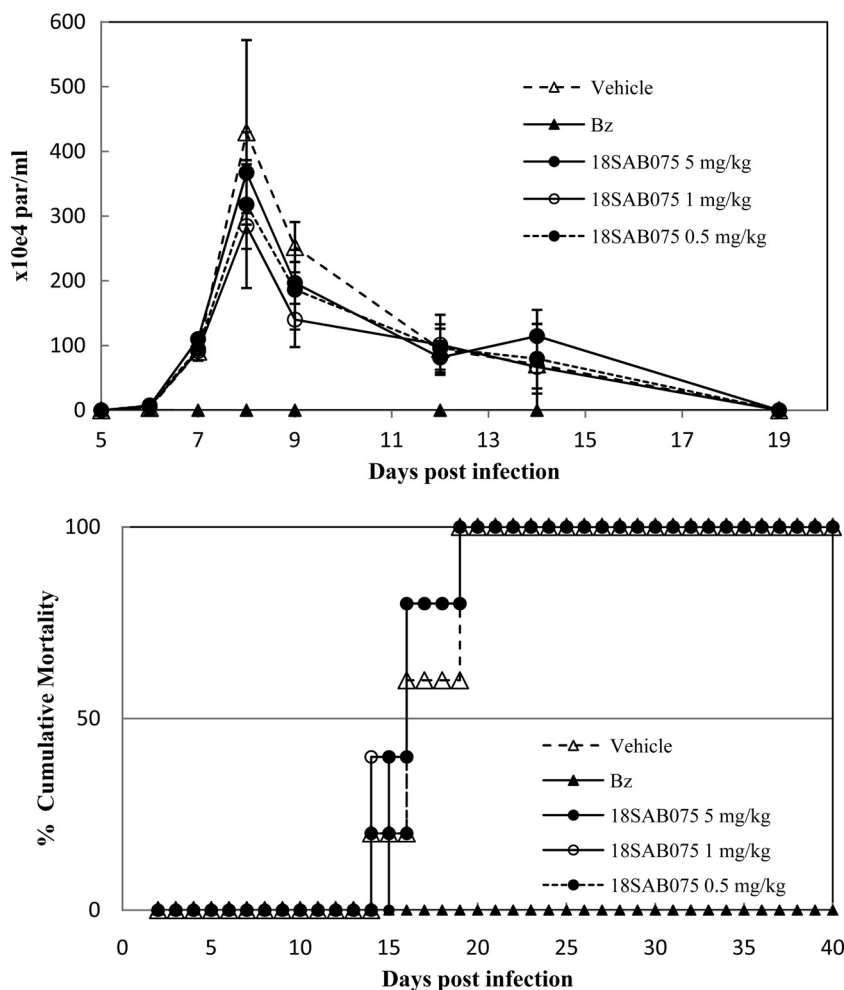


FIG 6 *In vivo* effect of 18SAB075 on *T. cruzi* infection using male Swiss mice inoculated with  $10^4$  bloodstream trypomastigotes (Y strain). The activities of 0.5, 1, and 5 mg/kg/day 18SAB075 (i.p.) and of benznidazole (100 mg/kg/day by gavage) given for 5 consecutive days (5 to 9 dpi) were evaluated through parasitemia levels (top) and cumulative mortality (bottom).

18SAB075 in all assayed therapeutic schemes (2 and 5 days of treatment) using nontoxic doses failed to demonstrate the activity of this diphenylacetylene AIA *in vivo*, while Bz not only suppressed the parasitemia but also induced 100% protection against mortality due to the experimental infection as described previously (26). Previous data using bis-AIAs like 2,5-bis[2-(2-propoxy)-4-(2-pyridylimino)aminophenyl]furan (DB 766) (15) and a related analog (DB 1831) (16) showed their high activity *in vivo* in the acute experimental mouse models of *T. cruzi*, exhibiting trypanocidal effects similar to that of Bz. In fact, with the same highly stringent experimental *in vivo* model as presently used for 18SAB075, the two previous studies reported that the presence of pyrimidine and pyridine units of bis-AIAs seemed to be advantageous for *T. cruzi* activity (14), supporting the continuity of preclinical studies of novel related molecules with the aim of finding new alternatives for treating Chagas disease.

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